A SIMPLE, RAPID MICROASSAY FOR DNA

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1. Introduction

There is a need for a more sensitive method for assaying DNA when attempting quantitative biochemical estimations on small numbers of cells. In the past, such measurements have been limited because it has been possible to express results only in terms of cell numbers or cellular protein. However, cell counting is tedious and often inaccurate due to clumping of cells, whilst protein content, for which the procedure of Lowry et al. [1] provides a sensitive assay, often bears little relationship to the number of cells present, especially in fibroblasts, differentiated cells and organ cultures.

When the supply of cells or tissue is adequate, and alternative approach is to measure the DNA content. Unfortunately, existing techniques for DNA estimations are laborious and often restricted by their sensitivity. For example, the Burton procedure [2] has a lower limit of about 10 μg or approximately 10^6 cells.

We have developed a rapid, sensitive and accurate assay for DNA in as few as 10^5 cells (or 0.5 μg of pure DNA). The method is based on the fact that the antibiotic mithramycin binds to double stranded DNA and fluoresces in direct proportion to the amount of DNA present [3–5]. This observation has previously [3] provided a basis for cell cycle analysis.

2. Methods

Mithramycin was kindly donated by Pfizer Limited for these studies as Mithracin, which contains mithramycin (1 part by weight) and mannitol and sodium diphosphate (40 parts by weight). A stock solution was prepared containing 200 μg/ml mithramycin and 300 mM $MgCl_2$, which promotes DNA-mithramycin interaction [4]. Bovine pancreatic DNase I, activity 2230 Kunitz units/mg, and yeast RNA type I, were purchased from Sigma. Bovine plasma albumin was obtained from Armour Pharmaceuticals.

Duplicate calf thymus DNA standards (Sigma, type I) were prepared using a final assay mixture containing DNA (0.2–16 μg/ml), mithramycin (10 μg/ml) and $MgCl_2$ (15 mM). The fluorescence of a 1 ml aliquot at 540 nm was determined using an Aminco-Bowmann spectrofluorometer at an excitation wavelength of 440 nm.

For investigations of the relationship between cell number and fluorescence in the presence of mithramycin, JI cells, a transformed line derived from baby hamster kidney cells were grown at 37°C in a humidified 5% CO_2–95% air atmosphere using Dulbecco's medium supplemented with 10% foetal calf serum. For estimation of cellular DNA, TA3B cells, a mouse tumour line, and human embryo lung fibroblasts were grown using Dulbecco's and Waymouth's medium respectively, supplemented with 10% newborn calf serum. After suspension of monolayer cultures by trypsinisation, the cells were centrifuged, resuspended in PBS (phosphate buffered saline) and counted in a haemocytometer. Duplicate aliquots of cell suspension were suspended in PBS to a final concentration of 10 μg/ml mithramycin and 15 mM $MgCl_2$. The fluorescence at 540 nm was determined as above, after sonication of the samples using an MSE ultrasonicator.

3. Results and discussion

Fig.1 shows that the mithramycin assay for DNA

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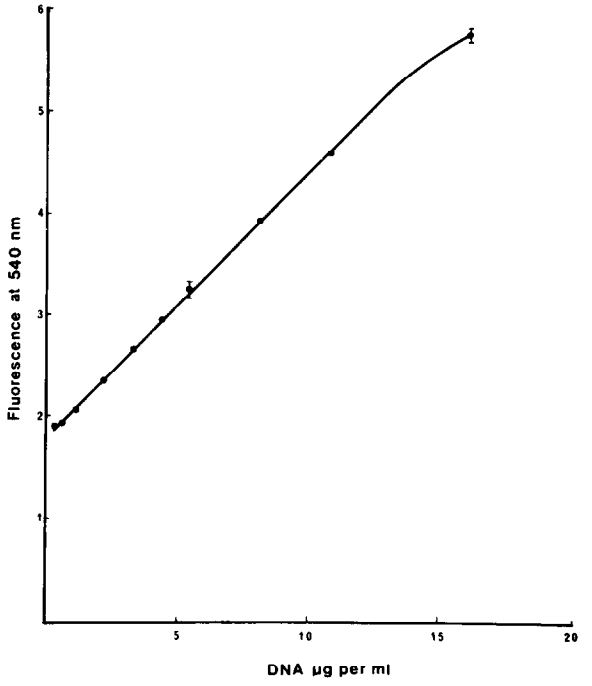

Fig.1. Linearity of mithramycin–DNA assay. The fluorescence of calf thymus DNA standards in duplicate, was determined at 540 nm after excitation at 440 nm.

is linear, sensitive and reproducible for as little as 0.5 µg/ml of DNA. The specificity of interaction between mithramycin and cellular DNA is supported by the lack of fluorescence in (i) deoxyribonuclease-treated DNA, (ii) acid hydrolysed DNA, (iii) RNA, and (iv) protein, and reduced fluorescence with heat-denatured DNA (see table 1), in agreement with earlier findings [3,5].

This technique was adapted to determine the DNA content of small numbers of cells. The cells used were mouse TA3B and JI's, a transformed line derived from baby hamster kidney cells, and human embryo lung fibroblasts. For the DNA assay, known numbers of cells were suspended in PBS containing 10 µg/ml mithramycin and 15 mM $MgCl_2$ and were disrupted by sonication prior to estimating the fluorescence of a 1 ml aliquot. Fig.2 shows that there is a relationship between the number of J1 cells and the fluorescence due to the mithramycin–DNA complex. This assay is accurate for as few as 10^5 cells/ml. Between 10^4–10^5 cells there is some degree of variation, which may be related to inaccurate cell sampling at these low numbers. Recovery of DNA after cell lysis by freezing and thawing, as opposed to sonication, provided less reproducible results. However, storage of cell pellets

Table 1
Specificity of the mithramycin–DNA microassay

Samples	Fluorescence values[a]
Mithramycin alone	100
DNA (8 µg/ml) + Mithramycin.	307
DNA (8 µg/ml) + DNAse (25 µg/ml) + Mithramycin, followed by incubation at 30°C for 120 min.	100
DNA (8 µg/ml) + DNAse (25 µg/ml) incubated at 30°C for 120 min followed by the addition of Mithramycin.	100
DNA (8 µg/ml) denatured by boiling + Mithramycin.	200
DNA (8 µg/ml) hydrolysed in 1 N PCA at 70°C for 30 min and neutralised prior to addition of Mithramycin.	100
RNA (190 µg/ml) + Mithramycin.	100
Protein (200 µg/ml) + Mithramycin.	110

[a] Fluorescence values are expressed as a percentage of those obtained with mithramycin alone.

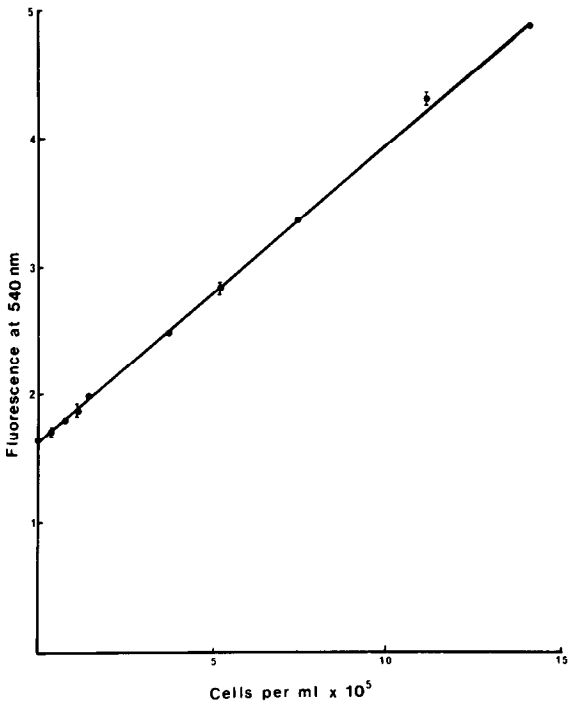

Fig.2. Relationship between cell number (JI cells) and fluorescence at 540 nm, after excitation at 440 nm.

at −20°C for 24–48 hr before the assay resulted in no significant loss of recovery of DNA and therefore multiple or serial samples may be collected and assayed when convenient.

Comparable results were obtained with TA3B cells and human embryo lung fibroblasts. Table 2 shows that the DNA content of TA3B and human embryo lung cells was 7.4–7.8 and 9.0–9.3 pg/cell respectively. These values are in good agreement with those obtained by the standard diphenylamine procedure, namely 8.2 and 9.1–9.5 pg/cell. The results for human cells agree well with those reported previously by other authors using the modified PCA-diphenylamine reaction [6], the ethidium bromide technique [7,8], a microspectrophotometric method [9], and estimations of the DNA phosphorus content [10,11].

This present method offers several advantages over existing techniques:

(i) The assay is rapid, providing an answer in minutes and requiring no overnight incubation as with the diphenylamine procedure,

(ii) The DNA content of as few as 10^5 cells can be determined accurately,

(iii) The assay is simple and multiple samples can be processed readily,

(iv) Samples can be stored conveniently as whole cell pellets at −20°C prior to assay.

(v) The assay is directly applicable to cell suspensions or monolayers. No time consuming or tedious extraction procedures are necessary, as is the case with the diphenylamine assay and the ethidium bromide technique.

(vi) The method is specific for DNA, and since there is no interference by RNA or protein, treatment with RNase and/or removal of

Table 2
Comparison of the levels of DNA in cells, using various assay procedures

Cell Type	DNA concentration (pg per cell)	
	Diphenylamine method	Mithramycin-DNA fluorescence
TA3B[a]	8.2 ± 0.5	7.8 ± 0.1
TA3B[b]	8.2 ± 0.6	7.4 ± 0.5
Human embryo lung fibroblasts[c]	9.5 ± 0.1	9.0 ± 0.4
Human embryo lung fibroblasts[d]	9.1 ± 0.2	9.3 ± 0.2

[a] The value obtained from 8 individual determinations of the same cell suspension.
[b] The value obtained from 4 determinations of 4 different cell suspensions.
[c] The value obtained from 4 individual determinations of the same cell suspension.
[d] The value obtained, as in [c] above, but after overnight storage at −20°C.

protein, as required in other methods is unnecessary.

(vii) Mithramycin, as Mithracin, is readily available and the method only involves the preparation of two water based solutions. The use of corrosive acids and inflammable liquids is avoided.

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